Chapter 4
Regulation of p27^{kip1} mRNA Expression by MicroRNAs

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Contents

Abstract p27^{kip1} (p27) is a cell cycle inhibitor and tumor suppressor whose expression is highly regulated in the cell. Low levels of p27 have been associated with poor prognosis in cancer. Recently, several microRNAs have been described to control p27 expression in various tumor types. In this chapter, we will provide an overview on the role of microRNAs in cancer, and will discuss how microRNAs regulate p27 expression and the implications for tumor progression.

4.1 Introduction

MicroRNAs (miRNAs) are conserved noncoding RNA molecules of about 22 nucleotides that regulate gene expression post-transcriptionally. Generally, miRNAs recognize complementary sequences present in multiple copies in the 3′ untranslated region (UTR) of target mRNAs and repress their translation (reviewed in Filipowicz et al. 2008). Translational repression is frequently accompanied by destabilization of the mRNA target, and, in some cases, mRNA degradation plays an important role in regulation (see Chap. 2 in this volume). Despite considerable efforts, the molecular

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mechanisms of translational repression by miRNAs are unclear at present. This aspect of miRNA regulation is discussed in detail elsewhere in this volume (see Chaps. 1 and 7 in this volume, chapter by Nilsen) as well as in a number of recent reviews (Standart and Jackson 2007; Jackson and Standart 2007; Eulalio et al. 2008; Filipowicz et al. 2008; Richter 2008), and will not be discussed further here.

Since their discovery, miRNAs have been shown to play a role in a wide variety of biological processes, including embryonic development, morphogenesis, proliferation, differentiation, inflammation, and apoptosis (reviewed in Bushati and Cohen 2007; Bueno et al. 2008). During these processes, miRNAs can act as rheostats that fine-tune the expression of many mRNAs in the cell or as robust regulators of specific target genes. One of these genes is the tumor suppressor $p27^{kip1}$ (p27), a cell cycle inhibitor whose deregulation contributes to tumor progression. In this chapter, we will focus on the role of miRNAs in cancer, and particularly on the expression of p27.

4.2 miRNAs in Cancer

Cancer is a complex genetic disease caused by accumulation of mutations leading to uncontrolled cell growth and proliferation. Classically, the causes of tumorigenesis have been attributed to alteration of protein-coding genes, but recent evidences indicate that changes in miRNA expression also contribute to tumor formation. For example, global depletion of miRNAs by impairing miRNA processing increases cellular transformation, and ectopic expression of miRNAs such as miR-155 or the miR-17-92 cluster accelerates tumor development (Kumar et al. 2007; He et al. 2005; Costinean et al. 2006). miRNAs can be used to distinguish normal from tumor tissue, cancer type, stage, and other clinical variables. In fact, profiling experiments have shown that miRNA changes are better predictors of tumor type than mRNA changes and have led to the identification of miRNA signatures for specific types of cancer (reviewed in Lee and Dutta 2009). miRNA expression profiles are not only a useful diagnosis but also a prognosis tool, as correlations have been established between the expression of certain miRNAs and the survival of patients (e.g., Yanaihara et al. 2006; Takamizawa et al. 2004). Multiple mechanisms underlie the widespread disruption of miRNA expression in tumors, including: (1) the alteration of the genomic region where miRNA genes are located (Calin et al. 2004); (2) the epigenetic modification – DNA methylation or histone deacetylation – of miRNA loci (Lehmann et al. 2008); (3) the aberrant transcription of miRNA precursors (He et al. 2007); and (4) the abnormal expression of factors involved in miRNA processing (Karube et al. 2005). In addition, the expression of modulators of miRNA function could also play a role (Kedde et al. 2007).

In order to understand the multiple functions of miRNAs in biological processes, it becomes essential to identify their targets. In silico predictions of miRNA targets are often inaccurate because the miRNA/mRNA interaction basically relies on a limited sequence length: the eight nucleotides of the miRNA seed region. In addition, mRNA recognition is influenced by the sequence context around the target site and

by factors that may block miRNA binding. Furthermore, although definitely useful, screenings based on over-expression of miRNAs usually yield a large number of false positives due to off-target effects. Alternative approaches consisting of immunoprecipitation of miRNP-associated transcripts or mRNA profiling after miRNA depletion are likely to improve the number of bona fide targets. To date, much information has accumulated about putative miRNA/mRNA pairs, but only a few have been experimentally validated. Validation is considered here in a rigorous sense, and includes reporter assays showing that the miRNA directly represses mRNA expression and that mutation of the miRNA binding sites abrogates regulation. Table 4.1 summarizes those validated pairs with a function in tumor development.

Table 4.1 Validated miRNA/ mRNA pairs in tumor progression

miRNA	mRNA target[a]	Mechanism[b]	Tumor	References
let-7	HMGA2	R	Ovary, lung	Shell et al. (2007) Lee and Dutta (2009) Mayr et al. (2007)
	NF2	nd	Cholangio carcinoma	Meng et al. (2007)
let-7g	k-Ras, c-Myc	nd	Various	Kumar et al. (2007)
miR-9,-125a, -125b	trkC	nd	Neuroblastoma	Laneve et al. (2007)
miR-10b	HOXD 10	T	Breast	Ma et al. (2007)
miR-16-1, -15a	Bcl-2	T	Leukemia	Cimmino et al. (2005)
miR-17-5p	AIB1	T	Breast	Hossain et al. (2006)
	p21	R	Neuroblastoma	Fontana et al. (2008)
miR-17-5p, -20	TβRII	R		Tagawa et al. (2007)
miR-20a	E2F1, 2, 3	T		Sylvestre et al. (2007)
miR-21	Pdcd4	T	Colon, breast	Asangani et al. (2007) Lu et al. (2008)
	TMP1	T		Zhu et al. (2007)
miR-29b	Mcl-1	T	Cholangio carcinoma	Mott et al. (2007)
miR-34a	Bcl-2	nd	Lung	Bommer et al. (2007)
miR-124a	CDK6	T	Lung, colon Medulloblastoma	Lujambio et al. (2007) Pierson et al. (2008)
miR-206	ERα	R	Breast	Adams et al. (2007)
miR-221,-222	p27	T	Glioblastoma, prostate	Le Sage et al. (2007) Galardi et al. (2007)
miR-372,-373	LATS2	T, R	Testis	Voorhoeve et al. (2006)
miR-378	Fus-1, Sufu	T	Glioblastoma	Lee et al. (2007)
BART cluster[c]	LMP1[(c)]	T	Nasopharynge	Lo et al. (2007)

[a]*HMGA2* High mobility group A2; *NF2* Neurofibromatosis 2; *trKC* tropomyosin-related kinase C; *AIB1* Amplified in breast cancer 1; *TβRII* Transforming growth factor beta-receptor type 2; *Pdcd4* Programmed cell death 4; *TMP1* Tropomyosin 1; *ERα* Estrogen receptor alpha; *LATS2* Large tumor suppressor homolog 2; *LMP1* Latent membrane protein 1

[b]*R* RNA degradation; *T* translational control; *nd* not determined

[c]Encoded by the EBV (Epstein–Barr) virus genome

miRNAs can either be up- or downregulated in tumors. let-7, one of the founding miRNA members, inhibits the expression of the oncogenes k-Ras, c-Myc, and HMGA2. Consistently, the levels of let-7 are reduced in several tumors, while they increase in differentiated tissues (e.g., Shell et al. 2007). Similarly, miR-15a and miR-16-1 regulate the expression of the mRNA encoding the antiapoptotic protein Bcl-2, and their levels are reduced in chronic lymphocytic leukemia (Cimmino et al. 2005). Conversely, the levels of the miR-17-92 cluster are high in primary neuroblastoma tumors, especially in those with poor prognosis. miR-17-92 inhibits the expression of the cell cycle inhibitor p21, leading to increased cell proliferation (Fontana et al. 2008). miR-10b and miR-21 are also over-expressed in cancer and promote invasion and metastasis via the translational repression of HOXD10, and TPM1 and PDCD4 mRNAs, respectively (Zhu et al. 2007; Lu et al. 2008; Ma et al. 2007). Interestingly, the related miRNA, miR-10a, also over-expressed in tumors, has been proposed to bind to the 5′ UTR of ribosomal protein mRNAs and to increase their translation, a function that may contribute to activate global protein synthesis and growth of transformed cells (Ørom et al. 2008). Thus, miRNAs can behave as either oncogenes or tumor suppressors depending on the cell type.

A recent screening has identified miR-221 and miR-222 as regulators of the expression of the tumor suppressor p27. The broad range of functions in which this protein is involved marks p27 as a prominent mediator of miRNA effects in cancer. In the following sections, we will discuss the roles of p27 and the implications of its regulation by miRNAs in tumorigenesis.

4.3 Role of p27 in Tumor Progression

The cell division cycle is driven by the alternate activity of cyclin-dependent kinases (CDKs). The activity of CDKs, in turn, is regulated by their association with regulatory cyclin subunits, the phosphorylation of their catalytic kinase subunits, their subcellular localization, and their binding to regulatory proteins called cyclin-dependent kinase inhibitors (CKIs) (reviewed in Malumbres and Barbacid 2007; Sherr and Roberts 2004). p27 is a member of the Cip/Kip family of CKIs; it binds into the catalytic cleft of the cyclin/CDK complex preventing ATP recognition (Russo et al. 1996). Typically, p27 inhibits the G1/S transition by binding to the S-phase promoting kinases, cyclin E/CDK2 and cyclin A/CDK2, although it has also been reported to inhibit the G2/M transition by regulating the activity of CDK1 (Aleem et al. 2005). The antiproliferative role of p27 depends on its localization in the nucleus, coincident with its target kinases. Cytoplasmic p27 performs alternative functions, including the regulation of cytoskeletal structure and cell migration. p27 inhibits the activity of the GTPase RhoA, which is necessary for the adhesion of cells to the substrate, thereby promoting cell motility (Besson et al. 2004). In addition, p27 is necessary for the complete differentiation of several cell types and has been shown to modulate apoptosis (e.g., Baldassarre et al. 1999; Nguyen et al. 2006; Bryja et al. 2004; Philipp-Staheli et al. 2001). The function of p27 in apoptosis appears

to be highly dependent on the experimental model, as some studies show a proapoptotic effect of the protein while others report an antiapoptotic role (reviewed in Borriello et al. 2007; Besson et al. 2008). It is interesting to note that the processes controlled by p27 (cell proliferation, differentiation, migration, and apoptosis) are often deregulated in cancer.

According to the important roles of p27 in the cell, expression of p27 is tightly regulated in a cell-type and condition-specific manner. The importance of maintaining adequate levels of p27 is illustrated by the phenotypes of the p27 knock-out and heterozygous mice. Null mice develop increased body size with multiple organ hyperplasia, show greater predisposition to induced tumorigenesis and develop pituitary tumors spontaneously (Fero et al. 1996; Kiyokawa et al. 1996; Nakayama et al. 1996). p27 heterozygous mice, containing half the amount of protein of wild type animals, show an intermediate phenotype (Fero et al. 1998). Molecular analysis of tumors from these mice showed that the remaining wild type allele was neither mutated nor silenced, indicating that p27 is haplo-insufficient for tumor suppression. p27 is also associated with spontaneous tumorigenesis in humans, since many human cancers express decreased amounts of p27 compared to normal tissues. Moreover, low levels of p27 frequently correlate with increased tumor aggressiveness and poor clinical outcome (Chu et al. 2008). Usually, the p27 gene is not mutated or deleted in cancer, but tumors associate with altered post-transcriptional regulation. Although in most cell types p27 plays an antitumorigenic role, elevated p27 levels are not always beneficial. Indeed, according to the function of p27 in cell migration, there is a positive correlation between elevated cytoplasmic p27 levels and invasiveness of a number of tumors, such as melanoma, leukemia, and breast, cervix, and uterus carcinomas (Denicourt et al. 2007; Dellas et al. 1998; Vrhovac et al. 1998; Kouvaraki et al. 2002; Watanabe et al. 2002). In addition, p27 might contribute to the resistance of some tumor cells to chemotherapy-induced apoptosis (Blain et al. 2003). Thus, given the complexity of p27 functions, an accurate knowledge of the mechanisms controlling p27 expression in each cell type is necessary to develop successful therapies against cancer.

4.4 Regulation of p27 Expression: Role of miRNAs

Expression of p27 is regulated at multiple levels, including transcription, mRNA stability, translation, proteolysis, and subcellular localization. Several mechanisms of regulation may coexist in a single cell depending on the cell type, the extracellular stimuli, and the biological circumstances (reviewed in le Sage et al. 2007b; Chu et al. 2008; Borriello et al. 2007; Vervoorts and Lüscher 2008; Koff 2006).

Translational regulation of p27 mRNA has emerged as a prominent mechanism to regulate p27 expression during differentiation, quiescence, and cancer progression. Early reports indicated that the translation of p27 mRNA increased in Hela cells arrested in G1 by treatment with lovastatin, in quiescent, contact-inhibited fibroblasts and in differentiated human promyelocytic leukemia (HL60) cells (Hengst and Reed

1996; Millard et al. 1997). Subsequent studies showed that the 5′ UTR of p27 mRNA contains a number of regulatory features that could allow p27 expression independently on the fate of most cellular mRNAs. For example, an upstream open reading frame was proposed to contribute to translational regulation of p27 during the cell cycle (Göpfert et al. 2003). In addition, several groups have reported the presence of an internal ribosome entry site (IRES) that promotes p27 translation in conditions where cap-dependent translation of most cellular messages is compromised (Miskimins et al. 2001; Kullmann et al. 2002; Cho et al. 2005; Jiang et al. 2007). The IRES is recognized by the proteins PTB, HuR, and hnRNPC1/C2 (Millard et al. 2000; Kullmann et al. 2002; Cho et al. 2005). However, the role of these proteins in p27 mRNA translation is unclear and the existence of the IRES has been recently disputed, as the detected activity was attributed to cryptic promoters in p27 5′ UTR (Liu et al. 2005; Cuesta et al. 2009).

More recently, miRNAs have appeared as chief regulators of p27 mRNA expression. The first indications that miRNAs could play a role in the expression of p27 were obtained in *Drosophila* (Hatfield et al. 2005). Inhibition of miRNA processing by mutation of dicer-1 resulted in delayed G1/S transition of germ-line stem cells, a process controlled by the p27 homolog Dacapo. Reduction of Dacapo levels partially rescued dicer-1 mutants and over-expression of Dacapo resembled dicer-1 mutations, suggesting that an adequate processing of miRNAs was necessary to repress Dacapo. Importantly, expression of a Dacapo transgene lacking a region of the 3′ UTR predicted to contain miRNA binding sites was not affected by dicer-1 mutations (Hatfield et al. 2005). These results suggested that the expression of Dacapo was regulated by miRNAs binding to the 3′ UTR. Indeed, mammalian p27 mRNA translation is regulated via its 3′ UTR in Hela, MDA468, and 3T3 cells (Millard et al. 2000; Vidal et al. 2002; Gonzalez et al. 2003). Significantly, similar to *Drosophila*, depletion of dicer in human glioblastoma cells increases p27 levels and decreases proliferation (Gillies and Lorimer 2007).

An elegant functional screening identified miRNAs that regulate p27 expression (le Sage et al. 2007a). In this screening, Hela cells expressing the GFP coding sequence fused to the 3′ UTR of p27 were transduced with a miRNA library, and cells expressing low levels of GFP were selected. The particular miRNA expressed in these cells was identified as miR-221. This miRNA also repressed the expression of endogenous p27, while p27 mRNA levels and the steady state of p27 protein remained unchanged. These results established that miR-221 represses the translation of p27 mRNA. Bioinformatics analysis predicted two target sites for miR-221 and the related miRNA miR-222 in the 3′ UTR of p27. miR-222 is encoded in the same genomic cluster as miR-221 and contains the same seed sequence. Validations using luciferase reporters showed that over-expressed miR-221/222 repressed the expression of transcripts containing wild type, but not mutated target sites. Conversely, mutation of the miRNA seed sequence in the miRNA- expressing vector abolished repression. In addition, antagomirs (antisense RNA oligos containing a molecule of cholesterol at the 5′ end and 2′-O-methylated at every nucleotide) against miR-221/222 inhibited proliferation of glioblastoma cells, whereas they had no effect on cell growth when p27 was depleted (le Sage et al. 2007a). These studies established a causal relationship

between miR-221/222, p27, and cell proliferation. miR-221/222 also regulate the expression of p27 in prostate carcinoma and melanoma cells, and their over-expression correlates with increased colony-forming potential and proliferation, respectively (Galardi et al. 2007; Felicetti et al. 2008). Collectively, the data suggest that miR-221/222 are oncogenes whose function is to repress the expression of the tumor suppressor p27 (Fig. 4.1).

The translation of p27 is also regulated by other miRNAs in different biological contexts. miR-181a was shown to repress the translation of p27 mRNA in undifferentiated HL60 cells (Cuesta et al. 2009). Repression by miR-181a is relieved during differentiation, allowing the accumulation of p27 necessary to fully block the cell cycle and reach the differentiated state. Intriguingly, one of the two target sites for miR-181a coincides with one of those binding to miR-221/222, suggesting that p27 3′ UTR contains hot-spots for miRNA-mediated regulation.

The modulation of miRNA/target interactions provides an additional level of plasticity to the regulation by miRNAs. Binding of miR-221/222 to their target sites in p27 3′ UTR can be blocked by the RNA-binding protein Dnd1 (Dead end 1), which recognizes U-rich sequences in the vicinity of the miRNA binding sites (Kedde et al. 2007). Dnd1 also counteracts the function of other miRNAs. Thus, cell proliferation and tumor progression should also be influenced by the relative amounts of Dnd1 and miRNAs, at least in primordial germ cells where Dnd1 is expressed.

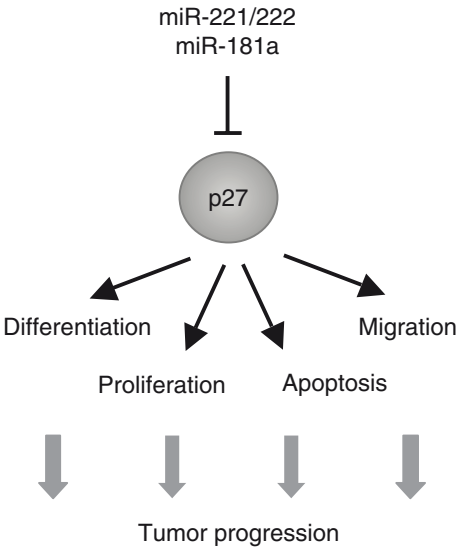

Fig. 4.1 Impact of the translational regulation of p27 mRNA. Translational control of p27 mRNA by miR-221/222 and miR-181a influences cell proliferation, differentiation, and apoptosis. Deregulated expression of miR-221/222 promotes tumor progression (see text for details)

4.5 Conclusions

p27 is a multifunctional protein that performs a dual role: in the nucleus, it acts as a tumor suppressor that inhibits cell proliferation by interfering with the activity of cyclin/CDK complexes; in the cytoplasm, it is an oncogene with prometastatic potential, in part due to its ability to regulate cell migration. Over the years, significant knowledge has accumulated about the mechanisms that regulate p27 protein degradation. Recently, translational regulation of p27 mRNA by miRNAs has emerged as a novel mode of control. Often, miRNAs repress translation only about 2-fold. Since p27 is haploinsufficient for tumor suppression, a reduction of the kind would be enough to promote tumor growth. miR-221 achieves this reduction in a number of tumors and, perhaps for this reason, upregulated miR-221 is part of a miRNA cancer signature. The regulation of p27 mRNA translation and stability are still largely unexplored. Learning about these mechanisms should greatly improve our capacity to develop successful therapies against cancer.

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